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ORIGINAL RESEARCH PAPER

Effect of the *C*-terminal domains and terminal residues of catalytic domain on enzymatic activity and thermostability of lichenase from *Clostridium thermocellum*

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Abstract To elucidate the effects of *C*-terminal domains of LicMB (mature lichenase from *Clostridium thermocellum*) and terminal residues of LicMB-CD (catalytic domain of LicMB) on the properties of lichenase, a series of truncated genes were constructed and expressed in *E. coli*. The Thr-Pro box had a positive effect while the dockerin domain had a negative impact on the properties of LicMB. The *N*-terminal 10–25th and *C*-terminal 1–9th residues of LicMB-CD were necessary to retain high thermostability while the *N*-terminal 1–7th and *C*-terminal 1–3rd residues were not necessary to maintain enzymatic activity.

Keywords *Clostridium thermocellum* · Lichenase · Thermostability · Truncated proteins

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Introduction

Lichenase (1,3–1,4-β-D-glucanase) hydrolyses only beta-1,4-linkages that are adjacent to beta-1,3-linkages in β-glucans, mainly producing cellobiosyltriose and cellotriosyltetraose (Fry et al. 2008). A thermostable lichenase from *Clostridium thermocellum* (*Lic*B) consists of four segments: (i) a signal peptide, (ii) a catalytic domain homologous to the full-length lichenase from *Bacillus*, (iii) a Thr-Pro box and (iv) a dockerin domain (Zverlov and Schwarz 2008). A modified lichenase consisting of a catalytic domain and part of the Pro-Thr box retained most of the activity and thermostability (Piruzian et al. 2002). However, there is very limited information regarding to the effects of LicB domains and terminal residues on its properties. Here we constructed a series of truncated enzymes and studied their catalytic activity and thermotolerance to clarify the thermostable mechanism of LicB.

Materials and methods

The recombinant plasmid pET-*Lic*B containing the *Lic*B gene from *Clostridium thermocellum* ZJL4 has been constructed in our laboratory. *E. coli* BL21 and pET-30a were purchased from Novagen, and all enzymes and reagents used in this experiment were from Takara.

Preparation, expression and purification of truncated enzymes

The truncated genes were constructed by PCR using pET-*Lic*B as template (primers are listed in Table 1). The recombinant *E. coli* BL21 were cultured in LB broth containing kanamycin (80 mg/l) at 37°C and induced by IPTG (1 mM), then disrupted by sonication (120 kW, 5 s, 20 times). The mixture was separated by $(NH_4)_2SO_4$ precipitation then desalted by a Sephadex G-25 column and finally purified by HiPrep16/10 Source 30S (GE Healthcare Bioscience).

Enzymatic activity and properties assay

The enzymatic activity was measured using a modified method based on Tezuka et al. (1989) and the detailed procedure was described in the footnote to Table 2. The optimal temperature and thermostablility of enzymes were determined under the conditions described in the footnotes to Tables 3, 4 and 5.

Results

All the truncated enzymes showed catalytic activities, indicating that all the mutated genes were successfully expressed in *E. coli*. The specific enzyme activity for LicMBΔ239-307 and Δ220-307 (equal to LicMB-CD) increased by 20 and 5.7%, respectively, compared to LicMB (mature lichenase from *C. thermocellum*) (490 U/mg protein). LicMB-CD, LicMB-CDΔN3, ΔN5, ΔN7 and ΔC3 showed similar levels of activity. When 9, 11, 13, 25 residues from the *N*-terminus and 6, 9 residues from the *C*-terminus were sequentially deleted, the values decreased dramatically (Table 2).

All the truncated enzymes possessed similar temperature profiles. The optimal temperature was 90°C for LicMBΔ239-307 and Δ220-307, and 80°C for LicMB and all the *N*-terminal truncated mutants except LicMB-CDΔN13 (70°C). The relative activities for LicMB-CD with deletion of 1–9th *N*-terminal residues retained more than 80% at 90°C and decreased significantly when more residues were deleted (Table 3).

Table 1 The primers of truncated mutants

Gene name	Forward primers	Reverse primers
*Lic*MB[a]	5′-tctagaaataatttgtttaactttaagaagga**gaattc**taatg*gaagccgcaactgtggtaaata*-3′[f]	5′-**gtcgac**ttaaagtgacggaattgcccg-3′
*Lic*MBΔ239-307[b]		5′-**gtcgac**ttagttagtcggagtagaaggag-3’
Δ220-307[c] or *Lic*MB-CD		
*Lic*MB-CDΔN3[d]	5′-**gaattc**taatggtaaatacgccttttgttgcag-3′	5′-**gtcgac**ttaaccgttaggatagtattttac-3′
ΔN5[d]	5′-**gaattc**taatgacgccttttgttgcagtgt-3′	
ΔN7[d]	5′-**gaattc**taatgtttgttgcagtgttttcgaact-3′	
ΔN9[d]	5′-**gaattc**taatggcagtgttttcgaactttgactc-3′	
ΔN11[d]	5′-**gaattc**taatgttttcgaactttgactccagtc-3′	
ΔN13[d]	5′-**gaattc**taatgaactttgactccagtcagt-3′	
ΔN25[d]	5′-**gaattc**taatggcgaacggttcggtgttc-3′	
ΔC3[e]	5′-**gaattc**taatggcaactgtggtaaata-3′	5′-**gtcgac**ttagtattttacatattcgtactc-3′
ΔC6[e]		5′-**gtcgac**ttaatattcgtactccgcctgcaa-3′
ΔC9[e]		5′-**gtcgac**ttactccgcctgcaaaggagttct-3′

[a] Mature lichenase from *Clostridium thermocellum*

[b] *Lic*MB with dockerin domain deleted

[c] *Lic*MB with both dockerin domain and Thr-Pro box deleted, i.e. catalytic domain

[d] *Lic*MB-CD with the *N*-terminal residues deleted

[e] *Lic*MB-CD with the *C*-terminal residues deleted

[f] The *lic*MB gene should be inserted in the *Nde*I site of pET-30a in order to be expressed in native form. As this gene also has an *Nde*I site, *Eco*RI was introduced to replace the *Nde*I in pET-30a

Restriction sites *Eco*RI (gaattc) and *Sal*I (gtcgac) were shown in bold, and the start or stop codon in underline

Table 2 The specific enzyme activity of terminal truncated mutants of LicMB-CD

Enzyme	LicMB-CD	ΔN3	ΔN5	ΔN7	ΔN9	ΔN11	ΔN13	ΔN25	ΔC3	ΔC6	ΔC9
Activity[a] (U/mg protein)	518 ± 19	552 ± 18	539 ± 15	495 ± 16	104 ± 8	27 ± 3	179 ± 12	17 ± 3	646 ± 22	85 ± 5	57 ± 4

Enzymatic activity assay procedure: 0.1 ml enzyme was added to 1 ml pre-incubated substrate solution (0.5 mg lichenin/ml, Sigma), then the mixture was incubated at 80°C for 10 min. The reaction was stopped by adding 0.5 ml reagent solution (10 g dinitrosalicylic acid, 2 g phenol, 0.5 g sodium sulfite, 10 g NaOH and 400 g potassium sodium tartrate in 1 l), and the amount of reducing sugar (glucose) was calculated by the absorption at 540 nm. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmo1 reducing sugar per min. Protein concentration was determined by Bradford method

[a] All the assays were repeated three times, and data were shown as means ± SD (this also applies to the following tables)

Table 3 Effect of temperature on activity of *N*-terminal truncated mutants of LicMB-CD

The truncated enzymes	The relative activity (%) at different temperatures (°C)[a]							
	37	40	50	60	70	80	90	100
LicMB-CD	26 ± 1	34 ± 1	51 ± 2	67 ± 2	84 ± 2	97 ± 3	100[b]	18 ± 1
ΔN3	33 ± 1	37 ± 1	58 ± 2	74 ± 2	97 ± 2	100[b]	95 ± 3	14 ± 1
ΔN5	22 ± 1	28 ± 1	45 ± 2	66 ± 1	86 ± 1	100[b]	91 ± 2	13 ± 1
ΔN7	25 ± 1	33 ± 1	49 ± 1	644 ± 2	86 ± 2	100[b]	94 ± 2	11 ± 1
ΔN9	14 ± 1	20 ± 1	19 ± 1	45 ± 2	90 ± 3	100[b]	82 ± 3	10 ± 1
ΔN11	12 ± 1	28 ± 1	29 ± 2	42 ± 2	76 ± 2	100[b]	63 ± 2	35 ± 1
ΔN13	35 ± 1	43 ± 2	64 ± 2	83 ± 2	100[b]	49 ± 1	18 ± 1	6 ± 1
ΔN25	28 ± 1	56 ± 1	61 ± 2	65 ± 2	91 ± 1	100[b]	47 ± 1	15 ± 1

[a] The enzymatic activity was measured by standard assay at different temperatures, and the relative activity was calculated by taking the maximum enzymatic activity as 100%. The maximum enzymatic activities for LicMB-CD, ΔN3, ΔN5, ΔN7, ΔN9, ΔN11, ΔN13 and ΔN25 were 534, 552, 539, 495, 104, 27, 365 and 17 U/mg protein, respectively

Table 4 The residual activity of three truncated lichenases

Enzyme	The residual activity (%)			
	75°C 10 min		80°C 5 min	
	Ca^{2+a}	No Ca^{2+b}	Ca^{2+a}	No Ca^{2+b}
LicMB	86 ± 2	51 ± 3	73 ± 1	54 ± 2
LicMBΔ239-307	100	79 ± 3	99 ± 3	66 ± 3
Δ220-307	79 ± 2	48 ± 2	70 ± 2	42 ± 2

Samples were incubated in 50 mM sodium phosphate buffer (pH 8.0) at 75°C for 10 min or 80°C for 5 min exactly, then the enzymatic activity was measured by standard assay at 80°C. The residual activity was calculated as a ratio to a sample without incubation. The enzymatic activities for LicMB, LicMBΔ239-307 and Δ220-307 without incubation were 490, 588 and 518 U/mg protein in Ca^{2+} free buffer, and 820, 984 and 867 U/mg protein in 1 mM Ca^{2+} buffer, respectively

[a] 1 mM $CaCl_2$ in sodium phosphate buffer

[b] No Ca^{2+} in buffer by addition of 2 mM EDTA

The thermostability of LicMBΔ239-307 was higher than that of LicMB and LicMBΔ220-307, and could be influenced by Ca^{2+} (Table 4). Compared to LicMB-CD, the residual activities for mutants with deletion of the *N*-terminal 1–9th residues slightly lowered, when more residues were deleted, the values decreased dramatically (Table 5). The LicMB-CDΔC3, ΔC6 and ΔC9 only retained 41, 24 and 8% residual activity, respectively, when incubated at 75°C for 5 min.

Discussion

The Thr-Pro box connects the catalytic and dockerin domains of LicB. Our results imply that the dockerin domain has a negative effect while the Thr-Pro box has a positive effect on the catalytic efficiency and

Table 5 The residual activity of the *N*-terminal truncated mutants of LicMB-CD

Temperature (°C)	Time (min)	LicMB-CD	ΔN3	ΔN5	ΔN7	ΔN9	ΔN11	ΔN13	ΔN25
50	10	93 ± 3	107 ± 3	100 ± 3	101 ± 2	96 ± 2	56 ± 1	100 ± 3	60 ± 1
	20	99 ± 2	100 ± 3	95 ± 3	113 ± 3	108 ± 3	39 ± 2	78 ± 2	46 ± 1
	30	98 ± 3	95 ± 3	90 ± 2	102 ± 3	117 ± 2	16 ± 1	67 ± 2	14 ± 1
60	10	97 ± 2	95 ± 2	92 ± 3	98 ± 2	108 ± 4	0	65 ± 1	18 ± 1
	20	100 ± 3	96 ± 2	102 ± 3	107 ± 3	107 ± 4	0	0	0
	30	104 ± 3	95 ± 2	96 ± 2	98 ± 2	96 ± 2	0	0	0
70	10	98 ± 2	88 ± 3	87 ± 3	85 ± 2	73 ± 3	0	0	0
	20	93 ± 3	92 ± 2	78 ± 2	75 ± 3	51 ± 1	0	0	0
	30	88 ± 2	1 ± 0	2 ± 0	4 ± 1	5 ± 0	0	0	0

Samples were incubated in 50 mM sodium phosphate buffer (pH 8.0) for 10, 20 and 30 min at 50, 60 and 70°C, respectively, then the enzymatic activity was measured by standard assay at 80°C, and the residual activity was calculated as a ratio to a sample without incubation. The enzymatic activities for LicMB-CD, ΔN3, ΔN5, ΔN7, ΔN9, ΔN11, ΔN13 and ΔN25 without incubation were 518, 552, 539, 495, 104, 27, 179 and 17 U/mg protein, respectively

thermo-tolerance of lichenase, which might be related to protein structural change (Pinheiro et al. 2008; Shen et al. 1991). However, these two *C*-terminal domains had little effect on the optimal temperature, indicating that deletion of them did not influence the conformation of enzyme-substrate complex. Ca^{2+} increased the thermostability of truncated lichenase which indicates that Ca^{2+} binding might stabilize the three-dimensional structure of lichenase (Keitel et al. 1994).

Our results showed that both the *N*- and *C*-terminal residues play important roles on properties of LicMB-CD. Deletion of the first 7 *N*-terminal residues did not affect its catalytic efficiency but did decrease the thermostability. Only one H-bond could be formed between these residues and their tertiary neighboring amino acids (≤4.0 Å) (Swiss-PDB viewer version 3.7). This bond might be essential to keep the whole structure of LicMB-CD to maintain thermostability but not to the active-site structure. Truncation of the *N*-terminal 8–13th residues would result in elimination of the four H-bonds formed between them and neighboring residues; when more residues were deleted more H-bonds and other interactions would disappear. The disruption of these interactions might cause the decreased enzymatic activity and thermostability. However, there was no change in the optimal temperature for all the *N*-terminal mutants except LicMB-CDΔN13, indicating interactions between the *N*-terminal 1–25th and their neighboring residues might have less effect on thermophily of LicMB-CD.

Two or sixteen H-bonds could be formed between the *C*-terminal 1–3rd or 4–9th and their tertiary neighboring residues, respectively. The deletion of these bonds might account for the decrease of thermostability for all the *C*-terminal truncated mutants and enzymatic activity for LicMB-CDΔC6 and ΔC9. Our results were in agreement with other reports, which stated that the change of H-bonds or other interactions between protein atoms could influence the properties of enzyme (Politz et al. 1993; Goda et al. 2000; Hamana and Shinozawa 1999; Wen et al. 2005). This work gives a support for the method of truncation/elongation in protein engineering (Matsuura et al. 1999; Cho et al. 2008).

In conclusion: The dockerin domain played a negative, while the Thr-Pro box a positive role on the properties of LicMB. The *N*-terminal 10–25th and *C*-terminal 1–9th residues were necessary to retain the high thermo-tolerance whereas the *N*-terminal 1–7th and *C*-terminal 1–3rd residues weren't to maintain the catalytic efficiency of LicMB-CD.

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